Towards the development of antistaphylococcal immunotherapy

Koedijk, Danny

Chapter 1

General introduction and scope of the thesis

The genus *Staphylococcus* consists of more than 40 Gram-positive coccoid bacterial species of which *S. epidermidis, S. haemolyticus* and *S. aureus* are the most abundant representatives in the microbiomes of humans and animals (1,2). In human beings, *S. aureus* is usually present as a commensal in the nostrils, but it can also be found in other niches, such as the oral cavity, the skin, the perineum and in the gastrointestinal tract. The group of persistent nasal carriers of *S. aureus* ranges between 12 and 30%, while the percentage of intermittent carriers seems to vary from 16 to 70% (3). In itself carriage of *S. aureus* is asymptomatic but, upon invasion of the body, this bacterium shows its true nature as a dangerous pathogen. To survive and thrive in the human body, *S. aureus* produces many virulence factors, including cell surface located proteins, which are responsible for the interaction with the host (4). Once the mucosal barrier or the skin are damaged, the bacterium can reach and infect almost every organ and tissue. This can cause mild, severe or even fatal infectious diseases, like sepsis, endocarditis, and osteomyelitis, if not treated properly.

Most infections caused by *S. aureus* are treated with antibiotics. In the 1940s the first antibiotic used for treatment was penicillin. After only two years of clinical use, *S. aureus* strains were detected that had developed resistance against penicillin (5,6). This history repeated itself upon the clinical introduction of the antibiotic methicillin in the 1960s, where resistance development was observed within a year (7) resulting in the currently well-known methicillin resistant *S. aureus* (MRSA). Since then, MRSA has become notorious for causing difficult-to-treat outbreaks in hospitals and the community (8). Over time other antibiotics, like vancomycin (2002) (9), linezolid (2001) (10), mupirocin (1985) (11), and daptomycin (2005) (12) were introduced to treat patients with MRSA infections but, unfortunately, the emergence of *S. aureus* lineages resistant against such new antibiotics has been observed within several years. Notably, antibiotic resistance frequently originates in hospitals where the selective pressure for resistance is the greatest. Subsequently, resistant bacteria may

spread into the community (8). As a consequence, multi-drug resistant *S. aureus* lineages and strains have now appeared in many places all over the world by transmission from individual to individual. According to a recent report of the World Health Organization, infections with antibiotic resistant microbes are expected to be the number one cause of death in 2050 (13; https://amr-review.org/), and MRSA has very recently been ranked in 5th position amongst the "WHO priority pathogens list" for the development of new antimicrobial therapies (http://www.who.int/mediacentre/news/ releases/ 017/ bacteria-antibiotics-needed/en/).

Immunotherapy is a classical alternative to the use of antibiotics for prevention or treatment of infections. Accordingly, active or passive immunization are also considered as alternative possibilities to prevent or treat infections with drug-resistant *S. aureus*. The results of studies in different animal models indicate that, indeed, vaccination or treatment with antibodies can also be applied successfully to fight *S. aureus* infections (14-17). In active immunization (i.e. vaccination), the induction of immunity after exposure to antigens of an inactivated or attenuated pathogen may result in the development of a long-lasting protective immune response. In contrast, in a passive immunization, a person is protected against a pathogen by administering specific antibodies that are nowadays in most cases produced biotechnologically. Good targets for active or passive immunization strategies need to have particular properties. First of all, the target needs to have antigenic determinants that provoke an effective immune response in the host. Factors exposed to the bacterial cell surface, such as polysaccharides and proteins, often comply with this requirement (18) and their efficacy can be enhanced with appropriate adjuvants. Furthermore, it is important that the targets are expressed at high levels by all lineages of a pathogen, and preferably they are also necessary for survival or fitness of this pathogen in the human body. The latter may preclude a so-called seroshift where pathogens stop producing the target and thereby evade the host's immune responses. Targets specifically involved in virulence, such as toxins, can also be attractive for immunization as the respective antibodies might neutralize the virulence function (19).

Like all Gram-positive bacteria, staphylococci are surrounded by a thick cell wall which serves as an exoskeleton that protects the bacteria against osmotic, chemical and mechanical insults from their environment (Fig. 1).

The main component of the cell wall is peptidoglycan (PG). Other components of the staphylococcal cell wall are proteins, lipo-teichoic acids (LTA), wall teichoic acids (WTA) and polysaccharides (4). Electron microscopic analysis showed that the *S. aureus* cell wall can be divided into an inner zone with a low-density and an outer zone of high-density (Fig. 1). This implies that there are areas of different composition within the cell wall, which possibly have different physical and chemical properties. It has been proposed that the low density inner wall zone mimics the periplasmic space present between the inner and outer membranes of Gram-negative bacteria (20). Notably, even though the cell wall is a thick and rigid structure, it is highly dynamic due to continuous degradation and biosynthesis that are needed in order to allow cells to grow and divide.

Specifically, the cell wall of *S. aureus* is made up of multiple PG layers of which the strands consist of alternating disaccharide *N*-acetylmuramic acid (β1-4)-*N*-acetylglucosamine (MurNac-GlcNac) units of different lengths that are stacked on top of each other (Fig. 1).

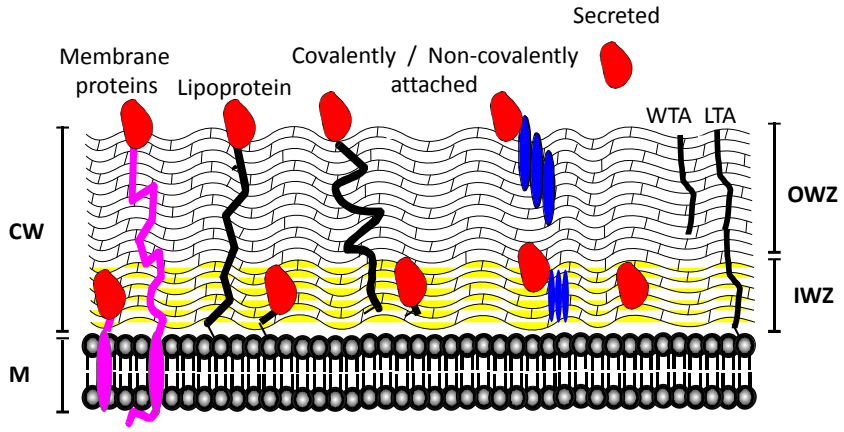

Figure 1. Schematic representation of exported proteins and their localization in the membrane (M), and the inner- (IWZ) or outer wall zones (OWZ) of the Gram-positive bacterial cell-wall (CW).

Proteins with one or more transmembrane-spanning domains can be inserted into the membrane. Lipoproteins are anchored to the membrane by a lipid modification. Proteins with wall retention signals are retained in the cell wall via covalent or high-affinity binding to cell-wall components, such as wall teichoic acids (WTA) or lipid teichoic acids (LTA). Exported proteins without a membrane or cell-wall retention signal can be secreted into the extracellular milieu (adapted from 22).

Glycan strands are one part of the three-dimensional structure of the cell wall, while the second part consists of cross-linked wall penta-peptides. These penta-peptides become linked via a penta-glycine cross bridge, while a D-Ala residue on position 5 of the second peptide is removed (21). Notably, cross-linking does not occur for all cell wall peptides. The cell wall peptides which are not cross-linked carry an extra D-alanine. LTA and WTA are cell wall glycopolymers, which are covalently attached to membrane glycolipids or PG, respectively. These long molecules have a negative charge, allowing ions to move through the cell wall. Besides peptidoglycan, (L)TAs and polysaccharides, the staphylococcal cell wall contains many different proteins. These proteins are exported from the cytoplasm and translocated across the bacterial C
cytoplasmic membrane via different specific export pathways (21,22). After passing the cytoplasmic membrane, the exported proteins can either be released (i.e. secreted) into the surrounding environment, or they may remain attached to the membrane or the cell wall by different mechanisms. Proteins that remain attached can be classified as membrane proteins, lipoproteins, and covalently or non-covalently attached proteins (Fig. 1). The membrane proteins and lipoproteins remain anchored to the cytoplasmic membrane by hydrophobic amino acid sequences (i.e. membrane anchors) or lipid modifications, respectively. Covalently cell wall-bound proteins contain an LPXT-G or NPQT-N motif which is cleft C-terminally of the Thr residue and connected to the peptidoglycan layer by sortase-mediated transpeptidation (21). The non-covalently cell wall-bound staphylococcal proteins contain particular domains that interact with specific cell wall components. Currently identified domains for non-covalent cell wall binding are the LysM motif (Lysin Motif, Pfam PF01476), and the SH3-5 (PF08460) and SH3-8 (PF13457) cell wall-binding domains. The LysM motif consists of 45 to 65 amino acid residues, which enable binding to the N-acetylglucosamine moiety of the peptidoglycan (23,24). The LysM motif is present in one or more copies in six different cell wall proteins of *S. aureus* (21,25). In contrast, SH3 domains bind to the staphylococcal pentaglycine interpeptide bridge (26,27). Collectively, the wall-located proteins that are exposed to the bacterial cell surface are referred to as the ‘surfacome’. These staphylococcal proteins can potentially serve as targets for antimicrobial therapy as they are easily accessible for drugs and antibodies. For this reason, the surfacome has become of major interest in the

development of novel passive or active immunotherapies against *S. aureus* (21,25).

To discriminate between wall-embedded and surface-exposed proteins, several different protein extraction approaches have been developed. For example, non-covalently attached proteins have been liberated from the cell wall by treatment of whole cells with $LiCl_2$, KSCN or SDS prior to their identification by proteomic techniques involving Mass Spectrometry (MS). Covalently attached proteins have been identified by isolation of cell wall fragments, removal of the non-covalently bound proteins and wall degradation with lysostaphin, followed by trypsin cleavage and identification by MS. The surface-exposed proteins or protein domains have been identified by surface shaving. The latter approach involved the incubation of cells with beads containing immobilized trypsin, and the subsequent collection and MS analysis of peptides liberated from the cell surface. Alternatively, surface-exposed proteins and protein domains were identified by a cross-linking approach with a membrane-impermeable biotinylated cross-linker, which allowed the affinity purification of biotinylated wall proteins from whole cells (28). Together, these approaches led to the identification of a fairly large number of different proteins associated with the staphylococcal cell wall (21,29,30). Not surprisingly, peptidoglycan hydrolases as listed in Table 1 form an important class of identified cell wall-associated proteins of *S. aureus* as such enzymes are responsible for the turnover of the Gram-positive bacterial cell wall during cellular growth and division.

As summarized in Table 1, several different studies identified the peptidoglycan hydrolases IsaA, Atl, SceD, Aly, LytM, Sle1 (also known as Aaa), SA0620 (SsaA-homolog), and SA2353 (SsaA1) as being localized to the *S. aureus* cell wall and/or surface. Interestingly, different peptidoglycan hydrolases were identified in different studies and also their identified relative abundances were variable (19,25,33,38,44,45). These differences may partly relate to the different approaches that were applied to identify these proteins. However, another explanation is that different studies were based on different *S. aureus* laboratory strains or clinical isolates, which is corroborated by comparative analyses in which different *S. aureus* strains were used (25,33). Overall, it appears from the so far published data that Atl, IsaA and LytM are among the most abundant surface-exposed peptidoglycan hydrolases of *S. aureus* with IsaA being most frequently identified (Table 1).

Table 1: Overview of surface-located and secreted peptidoglycan hydrolases of *S. aureus* identified using different types of proteomic techniques.

Name[1]	IsaA	Atl	SceD	Aly	LytM	Sle-1	SsaA homolog	SsaA1
GenBank[2]	SA2356	SA0905	SA1898	SA2437	SA0265	SA0423	SA0620	SA2353
Surface								
Becher 2009	■	■	■	■	■			
Dreisbach 2011	■	■						
Glowalla 2009	■	■	■	■	■			
Hempel 2011	■	■	■		■			
Liu 2014	■	■	■	■	■	■		■
Solis 2014	■	■	■	■	■	■	■	■
Ventura 2010	■	■		■		■		
Vytvytska 2002	■							
Secreted								
Enany 2012	■	■	■	■	■	■	■	
Kolata 2011		■				■		
Pasztor 2010	■	■		■				
Ravipaty 2010	■	■	■	■	■	■	■	
Rogasch 2006	■		■	■	■		■	
Ziebandt 2004	■		■	■	■		■	
Ziebandt 2010	■	■	■	■	■	■	■	

[1] Names of the identified peptidoglycan hydrolase, [2]Genbank numbers based on the *S. aureus* N315 genome annotation. In the first column, the proteomic studies that led to identification of the different *S. aureus* proteins at the cell surface or extracellular locations are listed. Of note, these studies were based on the following approaches: isolation of secreted proteins (Kolata 2011[31], Ziebandt 2004[32], 2010[33], Rogasch 2006[34], Enany 2012[35], Pasztor 2010[36], Ravipaty 2010[37]), isolation of surface proteins from cells using treatment with trypsin (Dreisbach 2011[21], Solis 2014[38], Ventura 2010[19]), lysostaphin (Vytvytska 2002[39]), urea or $LiCl_2$ (Liu 2014[40], Glowalla 2009[41]). Alternatively, cell wall proteins were identified by biotinylation (Becher 2009[42], Hempel 2011[28,44]).

It is presently not entirely clear why the cell surface proteome of *S. aureus* is so heterogeneous in this respect, but this variability may be part of this

pathogen's many strategies to evade the human immune system (25). Further, especially for studies carried out by different groups, one cannot exclude the possibility that variations relate to differences in growth, growth stage at the time of harvesting and other experimental conditions. This view is underpinned by a recent study where the *S. aureus* HG001 transcriptome was investigated under a range of different growth conditions, which showed substantial condition-dependent variations in genome-wide gene expression (46). On top of this, some strains appear to be more susceptible to cell lysis than others, which may lead to different outcomes in the proteomic analyses with respect to the extracellular identification of typical cytoplasmic proteins (43). Notably, data obtained from several studies showed that plasma samples from different individuals contained relatively high antibody titers against purified peptidoglycan hydrolases from *S. aureus*, especially IsaA, Atl, Aly, and LytM (47,48). Compared to plasma samples from healthy individuals, patients with the genetic blistering disease epidermolysis bullosa (EB) showed particularly high antibody levels against IsaA and LytM (47), suggesting that the respective antibodies might contribute to the apparent protection of these patients against invasive staphylococcal disease. In this respect it is noteworthy that patients with EB rarely suffer from invasive *S. aureus* infections despite the fact that they often have chronic wounds that are heavily colonized with this pathogen (49).

Scope of this thesis

The research described in the present thesis was aimed at exploring possibilities for future active or passive immunization approaches to protect frail individuals against infections with *S. aureus*. Particular attention was attributed to peptidoglycan hydrolases, like IsaA, LytM and Atl, as potential targets for immunotherapy in view of the fact that these enzymes were previously shown to elicit relatively high antibody titers in healthy individuals and EB patients. In this context it is also important to bear in mind that peptidoglycan hydrolases have critical roles in cell wall turnover, cell separation, antibiotic resistance, protein secretion, and virulence (50), which are additional features that make them interesting targets for novel immunotherapies. This general concept has been introduced in **Chapter 1** of

this thesis, and an outline for the respective investigations as presented in this thesis is schematically represented in Fig. 2.

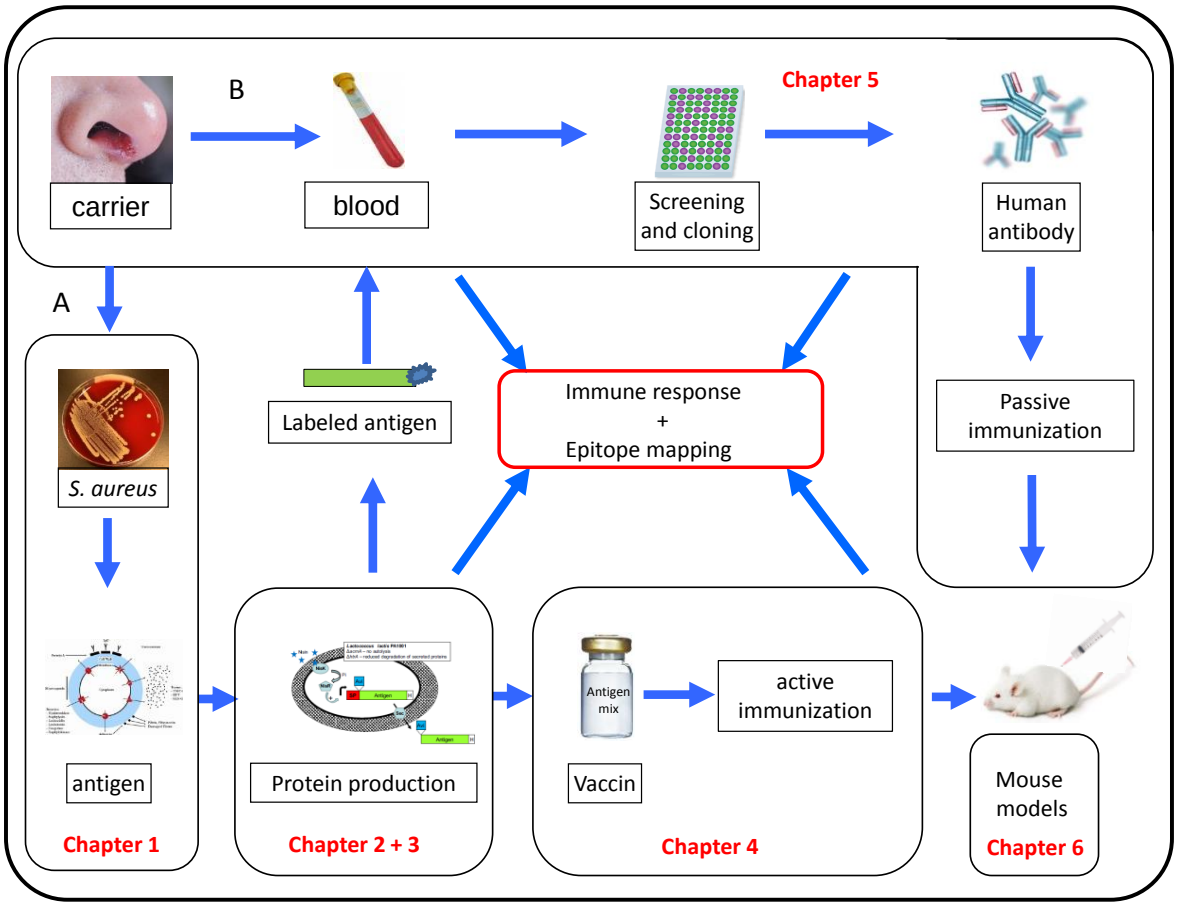

Figure 2. Schematic representation of the pipeline for the development and testing of passive and active immunization strategies against *S. aureus* infections in mouse models.
A. Route for the development of an active immunization strategy. Identification of invariant surface-exposed or secreted antigenic proteins from *S. aureus* isolates from healthy carriers or infected patients was achieved by proteomics. Subsequently, several of such antigens were produced in *Escherichia coli*- or *Lactococcus lactis*-based expression systems. Lastly, the purified recombinant antigens were tested, individually or in combination, for their potential use in active immunization against *S. aureus* infection using different mouse models.
B. Route for the development of a passive immunization strategy. B-cells producing antibodies against the *S. aureus* IsaA protein were isolated from blood of a healthy *S. aureus* carrier using the fluorescently labeled IsaA protein. Subsequently, immunoglobulins specific for IsaA were cloned and tested for protection against *S. aureus* infection in a mouse model. Lastly, the epitopes recognized by non-protective and potentially protective antibodies binding to IsaA were compared.

A first prerequisite for the development of active or passive immunization approaches is the identification and production of appropriate targets. In a previous study, the extracellular proteomes of 25 clinical *S. aureus* isolates from the University Medical Center Groningen were compared, which led to the identification of seven apparently invariant immunogenic determinants (33). Interestingly, five of these were peptidoglycan hydrolases, namely LytM, IsaA, Aly, SA0620 and SA2097. **Chapter 2** of this thesis describes the development and use of expression systems to produce these enzymes along with the invariant staphylococcal nuclease (Nuc). Specifically, the Gram-negative bacterium *E. coli* and the Gram-positive bacterium *L. lactis* were used for antigen production. The results show that *L. lactis* can be applied as an attractive production host for staphylococcal antigens, especially by setting limits to autolysis and product degradation. The latter was achieved by respectively deleting the *acmA* gene for the major autolysin of *L. lactis* and the *htrA* gene for the major extracellular protease of this bacterium. A clear advantage of the use of *L. lactis* was that the overproduced antigens can be secreted directly into the growth medium, allowing their easy one-step purification with the aid of a hexa-histidine tag (His_6-tag) and metal affinity chromatography. This technology was further enhanced by the development of an improved set of expression vectors as described in **Chapter 3**. These vectors facilitate the inducible secretory production of N- or C-terminally His_6-tagged proteins in *L. lactis*.

Chapter 4 reports on the application of purified *S. aureus* antigens in an active immunization approach. Specifically, an octa-valent antigen mixture containing three different peptidoglycan hydrolases (i.e. IsaA, Atl, LytM), Nuc, and the four phenol-soluble modulins α (PSMα1-4) of *S. aureus* was used to vaccinate mice. These antigens were selected, because they are invariantly produced by *S. aureus*, are immunogenic in humans, and have been implicated in staphylococcal virulence (21,33). Subsequently, protection against clinical methicillin sensitive *S. aureus* (MSSA) and MRSA was tested using murine bacteremia and skin infection models. The vaccinated mice showed high IgG responses against all antigens. However, compared to placebo-immunized mice, immunization with the octa-valent antigen mixture did not reduce the *S. aureus* load in blood, lungs, spleen, liver, and kidneys in either of the two

applied infection models. These findings show that the applied antigens are immunogenic in both humans and mice, but that the respective immune responses may not necessarily be protective against *S. aureus* infection.

In view of the fact that active immunization approaches as described in Chapter 4 have, thus far, not resulted in a protective vaccine against *S. aureus* infections, the possibility of developing a passive immunization approach was explored. To this end, IsaA was selected as a target. The results of this analysis are documented in **Chapter 5** of this thesis. Specifically, a fully human monoclonal antibody (huMab) named 1D9 was developed, which binds to IsaA with high affinity. In fact, 1D9 was shown to bind to all 26 clinical *S. aureus* isolates tested, including both MSSA and MRSA. Importantly, the prophylactic treatment with a single dose of 1D9 in a murine bacteremia model improved the survival of mice infected with the clinical *S. aureus* isolate P. On the other hand, therapeutic treatment with the same dose of 1D9 did not improve survival of the mice. Also, no protection was observed when the mice were challenged with the highly virulent community-associated *S. aureus* strain USA300. Altogether, this study shows that the huMab 1D9 is potentially protective against *S. aureus* infections, but that there is a need to further enhance its activity.

The finding that mice actively immunized with IsaA were not protected against *S. aureus* infection, whereas the anti-IsaA humAb 1D9 did provide some protection against *S. aureus* infection was apparently paradoxical (**Chapters 4** and **5**). Moreover, patients with the genetic blistering disease EB were previously shown to have high IsaA-specific IgG levels in their blood of which it was proposed that they may be protective against invasive staphylococcal diseases (47,49). To explain these differences in IsaA-specific immune responses and the possible protection against *S. aureus* infection, an epitope mapping analyses was performed as described in **Chapter 6** of this thesis. The results show that humAb 1D9 recognizes a conserved 62-residue N-terminal domain of IsaA and that the same region is recognized by the potentially protective IgGs from EB patients. In contrast, the non-protective anti-IsaA IgGs from mice immunized with IsaA were found to bind mostly to the C-terminal region of IsaA. Together, these findings imply that especially the N-terminal

region of IsaA could be a suitable target for future immunization approaches to protect patients against *S. aureus* infections.

Lastly, in **Chapter 7** the results described in this thesis are evaluated, and an outlook on future perspectives for the development of antistaphylococcal immunotherapy is presented.

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